L11: Entry 15 of 16 File: USPT Mar 19, 1985

DOCUMENT-IDENTIFIER: US 4506013 A
TITLE: Stabilizing and selecting recombinant DNA host cells

Brief Summary Paragraph Right (17):
Other plasmids can also be used to further exemplify the present invention. For example, the cro gene of bacteriophage lambda can be cloned onto plasmid pBR322 (disclosed in Bolivar, 1979, Life Sci. 25:807-818) by the insertion of the BamHI-EcoRI fragment of bacteriophage .lambda.cI857. The new plasmid, designated as pAR1, can be transformed into E. coli K12 RV308 and then the resulting strain can be lysogenized with bacteriophage .lambda.cI90. A similar operation can also be performed using E. coli K12 C600R.sub.k -M.sub.k - or E. coli K12 C600 as the host and bacteriophage .lambda.cI857 as the lysogenic organism. Since the .lambda.cro gene produces a repressor that replaces the function of the cI repressor, it is readily apparent that constructed strains E. coli K12 RV308.lambda.cI90/pAR1, E. coli K12 C600R.sub.k -M.sub.k -.lambda.cI857/pAR1 and E. coli K12 C600.lambda.cI857/pAR1 require the .lambda.cro containing plasmid for survival. However, since the .lambda.cI857 repressor is inactivated at 38.degree.-44.degree. C. or above (restrictive conditions) and is activated at lower temperatures (permissive conditions), the .lambda.cro containing plasmid is only required for survival in the latter strain under restrictive culture conditions. A comparison of plasmid retention in E. coli K12 C600R.sub.k -M.sub.k -.lambda.cI857/pAR1 under permissive condition and therefore without the present invention and under restrictive conditions and therefore with the present invention, clearly demonstrates that substantially all the viable cells in the culture with the invention have the desired plasmid. Also a comparison of plasmid retention in constructed strains E. coli K12 RV308.lambda.cI90/pAR1 with the invention and E. coli K12 RV308/pAR1 without the invention shows similar results. The use of plasmid pAR1 is particularly advantageous because the plasmid contains a promoter which is readily adaptable for the insertion of any one of a variety of genes coding for useful products.

Brief Summary Paragraph Right (26):
The .lambda.cI857 repressor gene used herein to illustrate the present invention is temperature sensitive and is inactivated at 38.degree. C. to 44.degree. C. or above. A temperature shift to 38.degree. C. to 44.degree. C. therefore lyses the cells by inducing the lytic cycle of the lambda prophage which, in accordance with the present invention, has been incorporated into the host cell strain. As is readily apparent, when a temperature sensitive repressor which represses a lethal or conditional lethal marker that causes host cell lysis is used and when the host cells are cultured at a temperature which inactivates the repressor and, in the case of a conditional lethal marker, at a temperature which is not within the temperature range for permissive culture of the host cells, the present invention also provides a simple, convenient, and inexpensive method to lyse cells for purification of intracellular products.

Brief Summary Paragraph Right (32):
Many classes of lethal mutations, identified as conditional lethal mutations, are expressed only under

restrictive conditions, such as for example, elevated temperature. Such mutations can be isolated and are lethal to cells when expressed but are not expressed or lethal under certain permissive culture conditions. Cell suicide of the present invention can be employed under restrictive conditions with any conditional lethal mutation so long as a plasmid, or other recombinant DNA cloning vector, carries an appropriate repressor which is functional under restrictive conditions. Such a mutation would not again become conditionally lethal unless the plasmid, or other recombinant DNA cloning vector, was lost.

Detailed Description Paragraph Right (5):
Fresh overnight cultures of E. coli K12 C600R.sub.K -M.sub.K - (disclosed in Chang and Cohen, 1974, Proc. Nat. Acad. Sci. 71:1030-1034) were subcultured 1:10 in fresh L-broth (disclosed in Miller, 1972, Experiments in Molecular Genetics, Cold Spring Harbor Labs, Cold Spring Harbor, N.Y.) and grown at 37.degree. C. for 1.0 hr. A total of 660 Klett units of cells were harvested, washed with 2.5 ml of 100 mM NaCl, suspended in 150 mM CaCl.sub.2 with 10.0% glycerol, and incubated at room temperature for 20 min. The cells were harvested by centrifugation, resuspended in 0.5 ml of CaCl.sub.2 -glycerol, chilled on ice for 3-5 minutes and frozen. The suspensions of cells were stored in liquid nitrogen until use. Preservation and storage did not adversely affect the viability or frequency of transformation by covalently closed circular DNA. The cells were thawed in an ice bath and mixed in a ratio of 0.1 ml of cells to 0.05 ml of DNA (prepared according to the teaching of Example 1) at a concentration of 2.0 .mu.g/ml. The samples thus prepared were chilled on ice for 10.0 minutes, and were then diluted with 0.85 ml of L-broth, incubated at 32.degree. C. for 2.0 hr, spread on L-agar (disclosed in Miller, 1972) with 5.times.10.sup.9 .lambda.b2, and incubated at 32.degree. C. Transformants were selected for immunity to bacteriophage .lambda.b2 at 32.degree. C. The recombinants were tested to verify Ap.sup.r, Tc.sup.s, .lambda.b2 immunity at 32.degree. C., and .lambda.b2 sensitivity at 42.degree. C. One transformant was selected and designated E. coli K12 C600R.sub.K -M.sub.K -/pAR2. This surviving colony was tested for the expected phenotypes and used for isolation and amplification of the constructed recombinant plasmid pAR2.

Detailed Description Paragraph Right (12):
Transformation of plasmid pAR1 into E. coli K12 C600R.sub.k -M.sub.k - is carried out according to the procedure taught in Example 2. Because the .lambda.cro repressor is not temperature sensitive, the transformants were selected for immunity to bacteriophage .lambda.b2 at both 32.degree. and 42.degree. C. The recombinants were further tested to verify Ap.sup.r and Tc.sup.s and one of the transformants was selected and designated E. coli K12 C600R.sub.k -M.sub.k -/pAR1. This surviving colony was tested for the expected phenotypes and was used for isolation and amplification of the recombinant plasmid pAR1. Both the isolation and amplification steps were carried out according to the procedure taught in Example 3.

Detailed Description Paragraph Right (24):
Plasmid pSOM7.DELTA.2 was HindIII digested followed by digestion with lambda exonuclease (a 5' to 3' exonuclease) under conditions chosen so as to digest beyond the BglII restriction site within the LE' encoding region. About 20 .mu.g of HindIII-digested pSOM7.DELTA.2 was dissolved in buffer (20 mM glycine buffer, pH 9.6, 1 mM MgCl.sub.2, 1 mM .beta.-mercaptoethanol). The resulting mixture was treated with 5 units of lambda exonuclease for 60 minutes at room temperature. The reaction mixture obtained was then phenol extracted, chloroform extracted, and ethanol precipitated.

Detailed Description Paragraph Right (26):
The reaction mixture was thus heated to 50.degree. C. and let cool slowly to 10.degree. C., whereafter 4 .mu.l of Klenow enzyme were added. After 15 minutes incubation at room temperature, followed by 30 minutes incubation at 37.degree. C., the reaction was stopped by the addition of 5 .mu.l of 0.25 molar EDTA. The reaction mixture was phenol extracted, chloroform extracted, and ethanol precipitated. The DNA was subsequently cleaved with the restriction enzyme BglII and the fragments were separated by PAGE. An autoradiogram obtained from the gel revealed a .sup.32 P-labelled fragment of the expected length of

approximately 470 bp, which was recovered by electroelution. As outlined, this fragment LE'(d) has a BglII terminus and a blunt end coinciding with the beginning of the primer.

Detailed Description Paragraph Right (31):
Trimers 1 and 3 (270 mg, 0.15 mmol; 145 mg, 0.075 mmol) were converted into their phosphodiesters (5 and 7) by treatment with triethylamine/pyridine/water (1:3:1, v/v, 10 ml) for 25 minutes at ambient temperature. Reagents were removed by rotary evaporation and the residues dried by repeated evaporations with anhydrous pyridine (3.times.10 ml). Trimer 8 (0.05 mmol) and trimer 7 were combined with TPSTe (50 mg, 0.15 mmol) in anhydrous pyridine (3 ml) and the reaction mixture left in vacuo at ambient temperature for two hours. TLC analysis showed that 95% of the trimer 8 had been converted into hexamer product (visualized by detection of the DMT group by spraying with 10% aqueous sulfuric acid and heating at 60.degree. C.). The reaction was quenched by addition of water (1.0 ml) and the solvent evaporated under reduced pressure. After removal of pyridine by coevaporations with toluene, the hexamer was deblocked at the 5' position with 2% BSA (8 ml) as described above for trimers 4 and 2. The product (10) was purified on a silica gel column (Merck 60 H, 3.5.times.5 cm) by step gradient elution with chloroform/methanol (98:2 to 95:5, v/v). Fractions containing product 10 were evaporated to dryness.

Detailed Description Paragraph Right (33):
Finally, hexamers 9 and 10 were coupled in anhydrous pyridine (2 ml) with TPSTe (75 mg, 0.225 mmol) as the condensing agent. Upon completion (4 hours, ambient temperature) the mixture was rotary evaporated and the residue chromatographed on silica gel. Product 11 (160 mg) was obtained by precipitation with petroleum ether and appeared homogeneous on TLC. A portion of compound 11 (20 mg) in pyridine (0.5 ml) was completely deblocked by treatment with concentrated ammonium hydroxide (7 ml, 8 hours, 60.degree. C.) and subsequent treatment in 80% acetic acid (15 minutes, ambient temperature). After evaporation of acetic acid, the solid residue was dissolved in 4% aqueous ammonium hydroxide (v/v, 4 ml) and extracted with ethyl ether (3.times.2 ml). The aqueous phase was concentrated to 1-2 ml and a portion applied to HPLC for purification of 12. The fractions corresponding to the major peak were pooled (ca. 2.0 O. D..sub.254 units) and concentrated to about 5 ml. The final product 12 was desalted on Bio-gel P-2 (1.5.times.100 cm) by elution with 20% aqueous ethanol, reduced to dryness and resuspended in water (200 .mu.l) to give a solution of A.sub.254 =10. The sequence of 12 was confirmed by two-dimensional sequence analysis.

Detailed Description Paragraph Table (2):
TABLE 1

SYNTHETIC OLIGONUCLEOTIDES FOR THYMOSIN.alpha.1 GENE HPLC Analysis Retention Time Compound Sequence Length (min)*

T.sub.1
A-A-T-T-C-A-T-G-T-C 10 17.4 T.sub.2 T-G-A-T-G-C-T-G-C-T-G-T-T-G-A 15 24.3 T.sub.3
T-A-C-T-T-C-T-TOC-T-G-A 12 20.3 T.sub.4 G-A-T-T-A-C-T-A-C-T-A-A-A 13 22.0 T.sub.5
G-C-A-G-C-A-T-C-A-G-A-C-A-T-G 15 24.8 T.sub.6 G-A-A-G-T-A-T-C-A-A-C-A 12 20.1 T.sub.7
A-G-T-A-A-T-C-T-C-A-G-A-A 13 22.6 T.sub.8 A-A-G-A-T-C-T-T-T-A-G-T 12 20.2 T.sub.9
G-A-T-C-T-T-A-A-G-G-A-G 12 20.4 .sup. T.sub.10 A-A-G-A-A-G- G-A-A-G-T-T 12 21.1 .sup. T.sub.11
G-T-C-G-A-A-G-A-G-G-C-T 12 20.5 .sup. T.sub.12 G-A-G-A-A-C-T-A-A-T-A-G 12 20.4 .sup. T.sub.13
C-T-T-C-T-T-C-T-C-C-T-T 12 19.9 .sup. T.sub.14 T-T-C-G-A-C-A-A-C-T-T-C 12 20.5 .sup. T.sub.15
G-T-T-C-T-C-A-G-C-C-T-C 12 20.2 .sup. T.sub.16 G-A-T-C-C-T-A-T-T-A 10 17.2

*at ambient temperature

CLAIMS:

1. A method for stabilizing and selecting bacterial host cells containing recombinant DNA which expresses a

functional polypeptide comprising:

(a) transforming the bacterial host cells with a recominant DNA cloning vector which contains both a repressor gene and a gene which expresses a functional polypeptide; and

(b) lysogenizing the transformed bacterial host cells with a bacteriophage lysogenic organism containing a marker which is lethal in the bacterial host cells but which is repressed in the transformed bacterial host cells by the repressor gene contained in the recombinant DNA cloning vector;

subject to the limitation that the recombinant DNA cloning vector contains a replicon and a promoter which are not sensitive to the repressor, and subject to the further limitation, that when the bacterial host cells are lysogenized with a bacteriophage lysogenic organism containing a gene which is conditionally lethal, the resulting bacterial host cells are cultured under restrictive conditions.

2. The method of claim 1 in which the recombinant DNA cloning vector is a plasmid.

3. The method of claim 1 which the recombinant DNA cloning vector is a bacteriophage.

4. The method of Claim 1 in which the gene which expresses a functional polypeptide is selected from the group consisting of genes coding for human insulin, human pre-proinsulin, human proinsulin, human insulin A-chain, human insulin B-chain, non-human insulin, human growth hormone, non-human growth hormone, human interferon, non-human interferon, viral antigen, urokinase, any polypeptide, any peptide hormone, and any peptide enzyme.

5. The method of Claim 1 in which the repressor gene is selected from the group consisting of chromosomal DNA replication mutation repressors, cell wall synthesis mutation repressors, ribosome mutation repressors, RNA polymerase mutation repressors, tRNA mutation repressors, amino acyl tRNA synthetase mutation repressors, cell division mutation repressors, and nonsense mutation repressors.

6. The method of Claim 1 in which the repressor gene is a cI repressor gene of bacteriophage lambda.

7. The method of Claim, 6 in which the cI repressor gene is cI857.

8. The method of Claim 1 in which the repressor gene is the .lambda.cro gene of bacteriophage lambda.

9. The method of Claim 1 in which the repressor gene is temperature sensitive and is inactivated at or above a temperature within a certain temperature range.

10. The method of Claim 9 in which the temperature range is 38.degree. C. to 44.degree. C.

11. The method of Claim 1 in which the lysogenic organism contains a bacteriophage .lambda.cI gene which does not produce a functional cI repressor.

12. The method of Claim 11 in which the lysogenic organism is bacteriophage lambda cI90.

13. The method of Claim 1 in which the lysogenic organism is bacteriophage .lambda.cI857.

14. The method of Claim 1 in which the bacteria are selected from the group consisting of E. coli, E. coli K12, E. coli K12 RV308, E. coli K12 C600R.sub.k -M.sub.k -, Bacillus, Bacillus subtilis, Staphylococcus, Streptococcus, Actinomycetes, Streptomyces, Serratia, Pseudomonas, and Agrobacterium.

15. The method of Claim 14 in which the bacteria are E. coli.

16. The method of Claim 14 in which the bacteria are E. coli K12.

17. The method of Claim 14 in which the bacteria are E. coli K12 RV308.

18. The method of Claim 14 in which the bacteria are E. coli K12 C600R.sub.k -M.sub.k -.

19. The method of claim 14 in which the bacteria are Streptomyces.

20. The method of claim 1 in which the recombinant DNA cloning vector is plasmid pAR2.

21. The method of claim 1 in which the recombinant DNA cloning vector is pAR1.

22. The method of claim 1 in which the transformed host cells are E. coli K12 RV308/pAR2.

23. The method of claim 1 in which the transformed host cells are E. coli K12 RV308/pAR1.

24. The method of claim 1 in which the tansformed host cells are E. coli K12 C600R.sub.k -M.sub.k -/pAR1.

25. The method of Claim 1 in which the lysogenized transformed host cells are E. coli K12 RV308.lambda.cI90/pAR2.

26. The method of Claim 1 in which the lysogenized transformed host cells are E. coli K12 RV308.lambda.cI90/pAR1.

27. The method of Claim 1 in which the lysogenized transformed host cells are E. coli K12 C600R.sub.k -M.sub.k -.lambda.cI857/pAR1.

28. A transformed bacterial host cell comprising:

(a) a recombinant DNA cloning vector containing both a repressor gene and a gene which expresses a functional polypeptide; and

(b) a chromosomal marker which is lethal or conditionally lethal but which is repressed by the repressor gene contained in the recombinant DNA cloning vector;

subject to the limitation that the recombinant DNA cloning vector contains a replicon and a promoter which are not sensitive to the repressor.

29. The transformed host cell of Claim 28 in which the recombinant DNA cloning vector is a plasmid.

30. The transformed host cell of Claim 28 in which the recombinant DNA cloning vector is a bacteriophage.

31. The transformed host cell of claim 28 in which the gene which expresses a functional polypeptide is selected from the group of genes consisting of naturally occurring genes, non-naturally occurring genes, and genes which are in part naturally occurring and are in part synthetic or non-naturally occurring.

32. The transformed host cell of claim 28 in which the gene which expresses a functional polypeptide is

selected from the group consisting of genes coding for human insulin, human pre-proinsulin, human proinsulin, human insulin A-chain, human insulin B-chain, non-human insulin, human growth hormone, non-human growth hormone, human interferon, non-human interferon, viral antigen, urokinase, any polypeptide, any peptide hormone, and any peptide enzyme.

33. The transformed host cell of claim 28 in which the repressor gene is selected from the group consisting of chromosomal DNA replication mutation repressors, cell wall synthesis mutation repressors, ribosome mutation repressors, RNA polymerase mutation repressors, tRNA mutation repressors, DNA restriction and modification mutation repressors, amino acyl tRNA synthetase mutation repressors, cell division mutation repressors, and nonsense mutation repressors.

34. The transformed host cell of claim 28 in which the repressor gene is a cI repressor gene of bacteriophage lambda.

35. The transformed host cell of claim 34 in which the repressor gene is cI857.

36. The transformed host cell of claim 28 in which the repressor gene is temperature sensitive and is inactivated at or above a temperature within a certain temperature range.

37. The transformed host cell of claim 36 in which the temperature range is 38.degree. C. to 44.degree. C.

38. The transformed host cell of Claim 28 in which the chromosomal marker is a bacteriophage .lambda.cI gene which does not produce a functional cI repressor.

39. The transformed host cell of Claim 38 in which the .lambda.cI gene is bacteriophage lambda cI90.

40. The transformed host cell of Claim 28 in which the chromosomal marker is bacteriophage .lambda.cI857.

41. The bacterium of Claim 38 which is selected from the group consisting of E. coli, E. coli K12, E. coli K12 RV308, E. coli K12 C600R.sub.k -M.sub.k -, Bacillus, Bacillus subtilis, Staphylococcus, Streptococcus, Actinomycetes, Streptomyces, Serratia, Pseudomonas, and Agrobacterium.

42. The bacterium of claim 41 which is E. coli.

43. The bacterium of claim 41 which is E. coli K12.

44. The bacterium of claim 41 which is E. coli K12 RV308.

45. The bacterium of claim 41 which is E. coli K12 C600R.sub.k -M.sub.k -.

46. The bacterium of claim 41 which is Streptomyces.

56. A method for lysing recombinant DNA containing bacterial host cells comprising:

(a) the method of claim 10 in which the repressor gene represses a marker that causes host cell lysis; and

(b) culturing the bacterial host cells at a temperature which inactivates the repressor and, in the case of a conditional lethal marker, at a temperature which is not within the temperature range for permissive culture of the host cells.

57. The method of claim 56 in which the temperature which inactivates the repressor is from 38.degree. to 44.degree. C.

58. The method of claim 56 in which the recombinant DNA cloning vector is a plasmid.

59. The method of claim 56 in which the recombinant DNA cloning vector is a bacteriophage.

61. The method of claim 56 in which the repressor gene is selected from the group consisting of chromosomal DNA replication mutation repressors, cell wall synthesis mutation repressors, ribosome mutation repressors, RNA polymerase mutation repressors, tRNA mutation repressors, amino acyl tRNA synthetase mutation repressors, cell division mutation repressors, and nonsense mutation repressors.

62. The method of claim 56 in which the repressor gene is a cI repressor gene of bacteriophage lambda.

63. The method of claim 62 in which the cI repressor gene is cI857.

64. The method of claim 56 in which the lysogenic organism contains a bacteriophage .lambda.cI gene which does not produce a functional cI repressor.

65. The method of claim 64 in which the lysogenic organism is bacteriophage lambda cI90.

66. The method of claim 56 in which the bacteria are selected from the group consisting of E. coli, E. coli K12, E. coli K12 RV308, E. coli K12 C600R.sub.k -M.sub.k -, Bacillus, Bacillus subtilis, Staphylococcus, Streptococcus, Actinomycetes, Streptomyces, Serratia, Pseudomonas, and Agrobacterium.

67. The method of claim 66 in which the bacteria are E. coli.

68. The method of claim 66 in which the bacteria are E. coli K12.

69. The method of claim 66 in which the bacteria are E. coli K12 RV308.

70. The method of claim 66 in which the bacteria are E. coli K12 C600R.sub.k -M.sub.k -.

71. The method of claim 66 in which the bacteria are Streptomyces.

72. The method of claim 56 in which recombinant DNA cloning vector is plasmid pAR2.

73. The method of claim 56 in which the transformed host cells are E. coli K12 RV308/pAR2.

74. The method of claim 56 in which the transformed host cells are E. coli K12 C600/pAR2.

75. The method of claim 56 in which the transformed host cells are E. coli K12 C600R.sub.k -M.sub.k -/pAR2.

76. The method of claim 56 in which the lysogenized transformed host cells are E. coli K12 RV308.lambda.cI90/pAR2.

77. The method of claim 56 in which the lysogenized transformed host cells are E. coli K12 C600R.sub.k -M.sub.k -.lambda.I90/pAR2.

78. The method of claim 56 in which the lysogenized transformed host cells are E. coli K12

C600.lambda.cI90/pAR2.

79. A method for lysing recombinant DNA containing bacterial host cells which comprises lysogenizing the bacterial host cells with a bacteriophage lysogenic organism containing a conditional lethal marker which causes host cell lysis and culturing the lysogenized bacterial host cells under restrictive conditions.

80. A method for lysing bacterial host cells which comprises transforming the bacterial host cells with a recombinant DNA cloning vector which contains a conditional lethal marker which causes host cell lysis and culturing the transformed bacterial host cells under restrictive conditions.

81. The method of claim 79 or 80 wherein the conditional lethal marker is bacteriophage .lambda.cI857.

82. The method of claim 1 in which the recombinant DNA cloning vector is plasmid pPR1.

83. The method of claim 1 in which the recombinant DNA cloning vector is plasmid pPR3.

84. The method of claim 1 in which the transformed host cells are E. coli K12 RV308/pPR1.

85. The method of claim 1 in which the transformed host cells are E. coli K12 RV308/pPR3.

86. The method of claim 1 in which the transformed host cells are E. coli K12 C600/pPR1.

87. The method of claim 1 in which the transformed host cells are E. coli K12 C600/pPR3.

88. The method of claim 1 in which the transformed host cells are E. coli K12 C600R.sub.k -M.sub.k -/pPR1.

89. The method of claim 1 in which the transformed host cells are E. coli K12 C600R.sub.k -M.sub.k -/pPR3.

90. The method of claim 1 in which the transformed host cells are E. coli K12 C600/pAR1.

91. The method of claim 1 in which the transformed host cells are E. coli K12 C600/pAR2.

92. The method of claim 1 in which the transformed host cells are E. coli K12 C600R.sub.k -M.sub.k -/pAR2.

93. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 RV308.lambda.cI90/pPR1.

94. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 RV308.lambda.cI90/pPR3.

95. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 C600.lambda.cI90/pPR1.

96. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 C600.lambda.cI90/pPR3.

97. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 C600R.sub.k -M.sub.k -.lambda.cI90/pPR1.

98. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 C600R.sub.k

-M.sub.k -.lambda.cI90/pPR3.

99. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 C600.lambda.cI857/pAR1.

100. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 C600.lambda.cI90/pAR2.

101. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 RV308.lambda.cI857/pAR1.

102. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 C600.lambda.cI90/pAR1.

103. The method of claim 1 in which the lysogenized transformed host cells are E. coli K12 C600R.sub.k -M.sub.k -.lambda.cI90/pAR1.

104. The method of claim 1 in which the host cells are E. coli K12 C600.

105. The bacterium of claim 28 which is E. coli K12 C600.

129. The method of claim 56 in which the recombinant DNA cloning vector is plasmid pPR1.

130. The method of claim 56 in which the recombinant DNA cloning vector is plasmid pPR3.

131. The method of claim 56 in which the lysogenized transformed host cells are E. coli K12 RV308.lambda.cI90/pPR1.

132. The method of claim 56 in which the lysogenized transformed host cells are E. coli K12 RV308.lambda.cI90/pPR3.

133. The method of claim 56 in which the lysogenized transformed host cells are E. coli K12 C600.lambda.cI90/pPR1.

134. The method of claim 56 in which the lysogenized transformed host cells are E. coli K12 C600R.sub.k -M.sub.k -.lambda.cI90/pPR3.

WEST Search History

updated 4/8/02

DATE: Monday, April 08, 2002

Set Name side by side	Query	Hit Count	Set Name result set
	DB=USPT; PLUR=YES; OP=AND		
L1	apoptosis	3363	L1
L2	L1 near5 (bacterial or bacterium or microorganism or micro-organism or gram or coli or salmonella or microbial or parasite or fungal or mycot$)	11	L2
L3	contain$.clm. and (lethal or sucid$).clm. and (heterolog$).clm.	2	L3
L4	(lethal or sucid$).clm.	475	L4
L5	L4 and (37 or degree or temperature)	372	L5
L6	L4 and (degree or temperature)	341	L6
L7	L4 and (temperature)	285	L7
L8	L7 and (cell or host or microb$ or bacteri$ or gram).clm.	128	L8
L9	L8 and (gene$ or nucleic or nucleotide or polynucleotide or poly-nucleotide or nuclear or dna or genetic or sequence)	116	L9
L10	L8 and (gene$ or nucleic or nucleotide or polynucleotide or poly-nucleotide or nuclear or dna or genetic or sequence).clm.	71	L10
L11	L10 and temperature.clm.	16	L11

END OF SEARCH HISTORY

WEST Search History

DATE: Monday, April 08, 2002

Set Name side by side	Query	Hit Count	Set Name result set
DB=USPT; PLUR=YES; OP=AND			
L1	apoptosis	3363	L1
L2	L1 near5 (bacterial or bacterium or microorganism or micro-organism or gram or coli or salmonella or microbial or parasite or fungal or mycot$)	11	L2
L3	contain$.clm. and (lethal or sucid$).clm. and (heterolog$).clm.	2	L3
L4	(lethal or sucid$).clm.	475	L4
L5	L4 and (37 or degree or temperature)	372	L5
L6	L4 and (degree or temperature)	341	L6
L7	L4 and (temperature)	285	L7
L8	L7 and (cell or host or microb$ or bacteri$ or gram).clm.	128	L8
L9	L8 and (gene$ or nucleic or nucleotide or polynucleotide or poly-nucleotide or nuclear or dna or genetic or sequence)	116	L9
L10	L8 and (gene$ or nucleic or nucleotide or polynucleotide or poly-nucleotide or nuclear or dna or genetic or sequence).clm.	71	L10
L11	L10 and temperature.clm.	16	L11
L12	permissive.clm. and (non-permissive or nonpermissive).clm.	47	L12
L13	(lethal or dead or kill$ or nonviable or non-viable or death or letha$).clm.	13565	L13

L14	L13 and l12	5	L14

END OF SEARCH HISTORY

WEST

Generate Collection Print

L11: Entry 2 of 16 File: USPT Feb 20, 2001

DOCUMENT-IDENTIFIER: US 6190666 B1
TITLE: DNA expression systems based on alphaviruses

Brief Summary Paragraph Right (49):
In said vaccine the chimaeric alphavirus is suitably attenuated by comprising mutations, such as the conditionally lethal SFV-mutation described before, amber (stop codon) or temperature sensitive mutations, in its genome.

Detailed Description Paragraph Right (20):
4. Immunofluorescence. To perform indirect immuno-fluorescence, infected cell monolayers on glass cover-slips were rinsed twice with phosphate-buffered saline (PBS) and fixed in -20.degree. C. methanol for 6 min. After fixation, the methanol was removed and the cover-slip washed 3 times with PBS. Unspecific antibody binding was blocked by incubation at room temperature with PBS containing 0.5% gelatin and 0.25% BSA. The blocking buffer was removed and replaced with same buffer containing primary antibody. After 30 min at room temperature the reaction was stopped by washing 3 times with PBS. Binding of secondary antibody (FITC-conjugated sheep anti-mouse [BioSys, Compiegne, France]) was done as for the primary antibody. After 3 washes with PBS and one rinse with water the coverslip was allowed to dry before mounting in Moviol 4-88 (Hoechst, Frankfurt am Main, FRG) containing 2.5% DABCO (1,4-diazobicyclo-[2.2.2]-octane).

Detailed Description Paragraph Right (24):
8. RNA transfection. Transfection of BHK monolayer cells by the DEAE-Dextran method was done as described previously (47). For transfection by electroporation, RNA was added either directly from the in vitro transcription reaction or diluted with transcription buffer containing 5 mM DTT and 1 unit/.mu.l of RNasin. Cells were trypsinized, washed once with complete BHK-cell medium and once with ice-cold PBS (without MgCl.sub.2 and CaCl.sub.2) and finally resuspended in PBS to give 10.sup.7 cells/ml. Cells were either used directly or stored (in BHK medium) on ice over night. For electroporation, 0.5 ml of cells were transferred to a 0.2 cm cuvette (BioRad), 10-50 .mu.l of RNA solution added and the solution mixed by inverting the duvette. Electroporation was performed at room temperature by two consecutive pulses at 1.5 kV/25 .mu.F using a BioRad Gene Pulser apparatus with its pulse controller unit set at maximum resistance. After incubation for 10 min, the cells were diluted 1:20 in complete BHK-cell medium and transferred onto tissue culture plates. For plaque assays, the electroporated cells were plated together with about 3.times.10.sup.5 fresh cells per ml and incubated at 37.degree. C. for 2 h, then overlayed with 1.8% low melting point agarose in complete BHK-cell medium. After incubation at 37.degree. C. for 48 h, plaques were visualized by staining with neutral red.

Detailed Description Paragraph Right (47):
BHK cells were transfected with the above SFV RNA molecules by electroporation and optimal conditions

were determined by varying parameters like temperature, voltage, capacitance, and number of pulses. Optimal transfection was obtained by 2 consecutive pulses of 1.5 kV at 25 .mu.F, under which negligible amounts of cells were killed. It was found that it was better to keep the cells at room tempeature than at 0.degree. C. during the whole procedure. Transfection by electroporation was also measured as a function of input RNA. As expected, an increase in transfection frequency was not linearly dependent on RNA concentration, and about 2 .mu.g of cRNA were needed to obtain 100% transfection.

Detailed Description Paragraph Type 0 (13):
9) Hahn, Y. S., Strauss, E. G., and Strauss, J. H. (1989b). Mapping of RNA-temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. J. Virol. 63, 3142-3150.

Detailed Description Paragraph Type 0 (19):
15) Hahn, Y. S., Grakoui, A., Rice, C. M., Strauss, E. G., and Strauss, J. H. (1989a). Mapping of RNA-temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4.

Detailed Description Paragraph Type 0 (20):
16) Sawicki, D. L., Barkhimer, D. B. Sawicki, S. G., Rice, C. M., and Schlesinger, S. (1990). Temperature sensitive shut-off of alphavirus minus strand RNA synthesis maps to a nonstructural protein, nsP4. Virology 174, 43-52.

Detailed Description Paragraph Type 0 (51):
47) Rice, C. M., Levis, R., Strauss, J. H., and Huang, H. V. (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: Mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J. Virol. 61, 3809-3819.

CLAIMS:

1. A recombinant RNA molecule which can be efficiently translated and replicated in an animal host cell, comprising an alphavirus RNA genome and an exogenous RNA sequence, wherein said alphavirus RNA genome contains at least one deletion or stop codon mutation such that at least one structural protein of the alphavirus cannot be made upon introduction of said recombinant RNA into said host cell, and further wherein said exogenous RNA sequence is operatively inserted into a region of the alphavirus RNA genome which is non-essential to replication of the recombinant RNA molecule such that the exogenous RNA is expressed from an alphavirus transcriptional promoter when the recombinant RNA is introduced into a host cell and further such that the exogenous RNA expresses its biological function in said host cell.

3. The recombinant RNA of claim 1, wherein the exogenous RNA sequence encodes a protein, a polypeptide or a peptide sequence defining an exogenous antigenic epitope or determinant.

4. The RNA recombinant of claim 3, wherein the exogenous RNA sequence encodes an epitope sequence of a structural protein of an immunodeficiency virus.

6. The recombinant RNA of claim 1, wherein the exogenous RNA sequence encodes a polypeptide or protein and is inserted into the subgenomic 26S RNA of said alphavirus by substituting a portion thereof.

7. The recombinant RNA of claim 6, wherein said exogenous RNA sequence is inserted into a portion of the 26S subgenomic RNA selected from the group consisting of a portion of the capsid protein RNA, the p62 RNA, the 6K RNA and the E1 RNA.

8. The RNA of claim 6, wherein the exogenous RNA sequence encodes a foreign viral epitopic peptide sequence and is operatively inserted into a portion of the subgenomic RNA coding for alphavirus structural proteins such that the exogenous RNA is expressed as an epitope constituting part of a matured recombinant virus particle.

9. The RNA of claim 6, wherein the exogenous RNA sequence encodes a foreign viral epitopic peptide and is inserted into the portion of the alphavirus genome encoding the p62 spike precursor subunit.

13. A DNA vector comprising a cDNA having one strand complementary to a recombinant RNA molecule that can be efficiently translated and replicated in an animal host cell, comprising an alphavirus RNA genome and an exogenous RNA sequence, wherein said alphavirus RNA genome contains at least one deletion or stop codon mutation such that at least one structural protein of the alphavirus cannot be made when the recombinant RNA is contained in said host cell, and further wherein said exogenous RNA sequence is operatively inserted into a region of the alphavirus RNA genome which is non-essential to replication of the recombinant RNA molecule such that the exogenous RNA is expressed from an alphavirus transcriptional promoter when the recombinant RNA is contained in said host cell and further such that the exogenous RNA expresses its biological function in said host cell.

14. The DNA vector of claim 13, further comprising a first promoter operatively linked to said cDNA, said promoter being operable in an animal cell such that transcription of said cDNA after introduction of said DNA vector into an animal cell produces a recombinant RNA molecule that can be efficiently translated and replicated in an animal host cell, said recombinant RNA comprising an alphavirus RNA genome and an exogenous RNA sequence, wherein said alphavirus RNA genome contains at least one deletion or stop codon mutation such that at least one structural protein of the alphavirus cannot be made when said recombinant RNA is contained in said host cell and further wherein said exogenous RNA sequence is operatively inserted into a region of the alphavirus RNA genome which is non-essential to replication of the recombinant RNA molecule such that the exogenous RNA is expressed under control of a alphavirus 26S promoter, when said DNA vector is introduced into a host cell and further such that the exogenous RNA expresses its biological function in said host cell.

15. The DNA vector of claim 14, further comprising a promoter for in vitro transcription of said cDNA, which is an SP6 promoter and said cDNA is located immediately downstream of the SP6 promoter and further wherein said cDNA has a 5'-terminal sequence of ATGG or GATGG and a 3'-terminal sequence of TTTCCA.sub.69 ACTAGT (SEQ ID NO: 25).

16. The DNA vector of claim 13, wherein the alphavirus transcriptional promoter is a Semliki Forest Virus promoter.

17. The DNA vector according to claim 14, wherein a portion of said cDNA encoding an alphavirus structural protein is deleted and further comprising a polylinker, wherein said polylinker is composed of DNA having a nucleotide sequence containing a plurality of restriction enzyme recognition sites.

18. The DNA vector according to claim 17, wherein said polylinker is operatively linked to said cDNA so as to allow expression of DNA encoding an exogenous protein in a host cell transformed with said DNA vector.

19. The DNA vector according to claim 18, wherein said restriction enzyme recognition sites are sites for the enzymes BamHI, SmaI and XmaI.

20. The DNA vector according to claim 18, wherein said polylinker is operatively linked to said cDNA so as to allow expression of DNA encoding an exogenous protein as a part of an alphavirus structural protein.

21. The DNA vector according to claim 20, wherein said polylinker is inserted into the region of the cDNA encoding the p62 spike protein.

22. The DNA vector according to claim 13, wherein said alphavirus cDNA contains a mutation in the protease cleavage site in the alphavirus structural protein homologous in function to the p62 protein of the Semliki Forest Virus, wherein said mutation results in expression of a p62-homologous protein that is not cleavable by intracellular proteases endogenous to said host cell.

23. The DNA vector according to claim 20, wherein said alphavirus cDNA contains a mutation in the protease cleavage site in the p62-homologous protein, wherein said mutation results in expression of a p62-homologous protein that is not cleavable by intracellular proteases endogenous to said host cell.

24. The DNA vector of claim 22, wherein the cell-entry activity of said p62-homologous protein can be activated by treatment with a protease in vitro.

25. The DNA vector of claim 24, wherein the cell-entry activity of said p62 protein can be activated by treatment with a protease in vitro.

26. The DNA vector of claim 25, wherein said protease is trypsin or chymotrypsin.

27. An RNA molecule made by transcription of the DNA vector of claim 13.

28. A method for producing recombinant alphavirus particles containing a recombinant alphavirus genome, comprising:

(a) producing a first and a second RNA transcript by in vitro transcription; wherein

(i) said first RNA transcript is made from a first vector comprising a promoter operatively linked to a cDNA encoding an alphavirus RNA that expresses at least one alphavirus structural protein and wherein said alphavirus RNA lacks sequences encoding RNA signals for packaging of RNA into alphavirus nucleocapsid particles, but contains the 5' and 3' nucleotides needed for replication of the alphavirus RNA in a host cell and also contains nucleotides encoding a promoter for expression of said RNA encoding said alphavirus structural protein when said first RNA transcript is contained in said host cell;

(ii) said second RNA transcript is made from a second vector comprising a promoter operatively linked to a cDNA encoding a recombinant alphavirus RNA genome, wherein said recombinant alphavirus RNA genome contains at least one deletion or stop codon mutation in the region encoding said structural protein encoded by said first vector, such that said structural protein that is encoded by said first vector cannot be made when said second RNA transcript is contained in said host cell, and encoding all other structural proteins necessary for assembly of an alphavirus particle, so that said other structural proteins are expressed in said host cell, and further wherein an exogenous RNA sequence, encoding said exogenous protein, is operatively inserted into a region of the recombinant alphavirus RNA genome such that the exogenous RNA expresses said exogenous protein in said host cell;

(b) transfecting a host cell with said first and second RNA transcripts produced in step (a) and allowing assembly of said recombinant alphavirus particles from structural proteins expressed from said first and second RNA transcripts; and

(c) recovering said recombinant alphavirus particles from cultures of said host cell.

29. A method for producing an exogenous protein, which comprises:

infecting a host cell with a recombinant alphavirus particle produced according to claim 28;

culturing said infected host cells; and

recovering said exogenous protein from the culture.

30. A cell containing a DNA vector according to claim 13.

31. A cell containing a DNA vector according to claim 14.

32. A cell containing a DNA vector according to claim 21.

33. The cell according to claim 30, which is a stably transformed animal cell.

34. The cell according to claim 33, wherein said animal cell is a BHK cell.

35. The cell according to claim 31, which is a stably transformed animal cell.

36. The cell according to claim 35, wherein said animal cell is a BHK cell.

37. A recombinant alphavirus comprising an alphavirus structural protein containing an amino acid sequence which is exogenous to said alphavirus said sequence being identical to a portion of the envelope glycoprotein of HIV.

39. The recombinant alphavirus according to claim 37, wherein said exogenous amino acid sequence comprises amino acids 309-325 of the envelope glycoprotein of HIV.

40. The recombinant alphavirus according to claim 37, wherein said exogenous amino acid sequence is inserted into the structural protein homologous in function to the p62 protein of Semliki Forest Virus.

41. A method for producing an antigen in an animal host cell maintained in cell culture or in an animal host comprising infecting said animal host cell with a recombinant alphavirus comprising a recombinant RNA according to claim 1, an alphavirus nucleocapsid and a surrounding membrane, wherein said membrane includes an alphavirus spike protein; and

recovering said antigen from said cell culture or from tissue or a fluid secreted from said animal host.

42. A method for producing an antiserum in vivo which comprises infecting an animal host with a recombinant alphavirus comprising a recombinant RNA according to claim 1, an alphavirus nucleocapsid and a surrounding membrane, wherein said membrane includes an alphavirus spike protein; and

recovering blood serum from said animal host.

44. A recombinant alphavirus comprising a recombinant alphavirus genome encoding an amino acid sequence exogenous to the wild-type of said alphavirus, wherein said exogenous amino acid sequence comprises an antigenic epitope or determinant inserted into the structural protein homologous in function to the p62 spike protein of Semliki Forest Virus.

45. A binary vector system comprising a recombinant RNA molecule which can be efficiently translated and replicated in an animal host cell, comprising an alphavirus RNA genome and an exogenous RNA sequence, wherein said alphavirus RNA genome contains at least one deletion or stop codon mutation such that at least one structural protein of the alphavirus cannot be made upon introduction of said recombinant RNA into said host cell, wherein said exogenous RNA sequence is inserted at said deletion or downstream from said stop codon, and further wherein said exogenous RNA sequence is operatively inserted into a region of the alphavirus RNA genome which is non-essential to replication of the recombinant RNA molecule such that the exogenous RNA is expressed from an alphavirus transcriptional promoter when the recombinant RNA is introduced into a host cell and further such that the exogenous RNA expresses its biological function in said host cell; and

a second vector encoding a conditional-lethal mutant of said at least one structural protein of the alphavirus, wherein said second vector expresses the conditional-lethal mutant of said at least one structural protein of the alphavirus upon introduction of the second vector into said host cell..

46. A binary vector system comprising a first recombinant DNA vector which encodes an RNA molecule which can be efficiently translated and replicated in an animal host cell, comprising an alphavirus RNA genome and an exogenous RNA sequence, wherein said alphavirus RNA genome contains at least one deletion or stop codon mutation such that at least one structural protein of the alphavirus cannot be made when said recombinant RNA is contained in said host, wherein said exogenous RNA sequence is inserted at said deletion or downstream from said stop codon, and further wherein said exogenous RNA sequence is operatively inserted into a region of the alphavirus RNA genome which is non-essential to replication of the recombinant RNA molecule such that the exogenous RNA is expressed from an alphavirus transcriptional promoter when the recombinant DNA is introduced into a host cell and further such that the exogenous RNA expresses its function in said host cell; and

a second recombinant DNA vector encoding a conditional-lethal mutant of said at least one structural protein of the alphavirus, wherein said second vector expresses the conditional-lethal mutant of said at least one structural protein of the alphavirus upon introduction of the second vector into said host cell.

47. The binary vector system according to claim 45, wherein said conditional-lethal mutant is a temperature-sensitive mutant.

48. The binary vector system according to claim 46, wherein said conditional-lethal mutant is a temperature-sensitive mutant.

49. A binary vector system comprising a recombinant RNA molecule which can be efficiently translated and replicated in an animal host cell, comprising an alphavirus RNA genome and an exogenous RNA sequence, wherein said alphavirus RNA genome contains at least one deletion or stop codon mutation such that at least one structural protein of the alphavirus cannot be made upon introduction of said recombinant RNA into said host cell, wherein said exogenous RNA sequence is inserted at said deletion or downstream from said stop codon, and further wherein said exogenous RNA sequence is operatively inserted into a region of the alphavirus RNA genome which is non-essential to replication of the recombinant RNA molecule such that the exogenous RNA is expressed from an alphavirus transcriptional promoter when the recombinant RNA is introduced into a host cell and further such that the exogenous RNA expresses its biological function in said host cell, and wherein said recombinant RNA molecule further contains a conditional-lethal mutation in at least one alphavirus protein encoded by said recombinant RNA molecule; and

a second vector encoding those wild-type structural proteins of the alphavirus not expressed as a result of

said deletion or stop codon mutations, wherein said second vector expresses said wild-type structural proteins of the alphavirus upon introduction of the second vector into said host cell.

50. The binary vector system according to claim 49, wherein said conditional-lethal mutant is a temperature-sensitive mutant.

51. A recombinant RNA molecule which can be efficiently translated and replicated in an animal host cell, comprising an alphavirus RNA genome and an exogenous RNA sequence, wherein said exogenous RNA sequence is inserted into a deletion in the alphavirus RNA genome, and further wherein said exogenous RNA sequence is operatively inserted into a region of the alphavirus RNA genome which is non-essential to replication of the recombinant RNA molecule such that the exogenous RNA is expressed from an alphavirus transcriptional promoter when the recombinant RNA is introduced into a host cell and further such that the exogenous RNA expresses its biological function in said host cell.

53. A method for producing a protein, an enzyme, a polypeptide antigen or a polypeptide hormone in a host cell maintained in a cell culture or in an animal host comprising:

i) introducing into said host cell the DNA vector of claim 14;

ii) effecting by step i) the transcription of said DNA vector to produce a recombinant RNA molecule comprising an alphavirus genome and an exogenous RNA sequence encoding said protein, enzyme, polypeptide antigen or polypeptide hormone, wherein said recombinant RNA molecule is subsequently translated and replicated in said host, to produce said protein, enzyme, polypeptide antigen or polypeptide hormone; and

iii) recovering said protein, enzyme, polypeptide antigen or polypeptide hormone from said host cell culture or from tissue of or a fluid secreted from said animal host.

54. A method for producing a protein, an enzyme, polypeptide antigen or a polypeptide hormone, in a host cell maintained in culture or in an animal host comprising:

i) introducing into said host cell a recombinant alphavirus comprising a recombinant RNA according to claim 1, an alphavirus nucleocapsid and a surrounding membrane, wherein said membrane includes an alphavirus spike protein, and further wherein said exogenous RNA encodes said protein, enzyme, polypeptide antigen or polypeptide hormone;

ii) effecting by step i) replication of said recombinant RNA and translation of said recombinant RNA in said host to produce said protein, enzyme, polypeptide antigen or polypeptide hormone; and

iii) recovering said protein, enzyme, polypeptide antigen or polypeptide hormone from said host cell culture or from tissue of or a fluid secreted from said animal host.

55. A method for producing a protein, an enzyme, polypeptide antigen or polypeptide hormone in a host cell maintained in culture or in an animal host comprising:

i) introducing into said host cell a recombinant RNA molecule according to claim 1, wherein said exogenous RNA encodes said protein, enzyme, polypeptide antigen or polypeptide hormone;

ii) effecting by step i) the replication of said recombinant RNA and the translation of said recombinant RNA to produce said protein, enzyme, polypeptide antigen or polypeptide hormone; and

iii) recovering said protein, enzyme, polypeptide antigen or polypeptide hormone from said host cell culture or from tissue of or a fluid secreted from said animal host.

56. An RNA vector comprising (i) a protein-coding ribonucleotide sequence that encodes a wild-type alphavirus structural protein or a conditional-lethal mutant thereof, (ii) 5' and 3' ribonucleotide sequences encoding signals for replication of an alphavirus RNA in a host cell and (iii) a ribonucleotide sequence functional as a promoter for transcription of said protein-coding ribonucleotide sequence in a host cell; wherein said RNA vector lacks ribonucleotide sequences encoding RNA signals for packaging of RNA into alphavirus particles.

57. A method for inducing an immune response in a subject comprising administering to said subject a chimeric alphavirus particle wherein said recombinant alphavirus particle comprises a chimeric alphavirus envelope protein comprising an immunogenic exogenous amino acid sequence inserted into the amino acid sequence of an envelope protein of said alphavirus.

58. A method for inducing an immune response in a subject comprising administering to said subject a composition comprising a recombinant RNA molecule which comprises (a) an RNA molecule genome that contains at least one deletion or stop codon mutation such that at least one structural protein of the alphavirus cannot be made upon introduction of said recombinant RNA molecule into a cell of said subject, and (b) an exogenous RNA sequence, wherein said exogenous RNA sequence is operatively inserted into a region of the alphavirus RNA genome which is non-essential to replication of the recombinant RNA molecule such that the exogenous RNA is expressed from an alphavirus transcriptional promoter when the recombinant RNA molecule is introduced into a cell of said subject and further such that the exogenous RNA expresses its biological function in said cell of said subject; thereby introducing said recombinant RNA molecule into a cell of said subject and eliciting an immune response in said subject.

60. The method of claim 58, wherein said recombinant alphavirus expresses a chimeric alphavirus envelope protein, said chimeric alphavirus envelope protein comprising an immunogenic exogenous amino acid sequence inserted into the amino acid sequence of an envelope protein of said alphavirus.